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Authors

Shi, W
Fan, H
Shum, L
[et al.](#)

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The Tetraspanin CD9 Associates with Transmembrane TGF- α and Regulates TGF- α -induced EGF Receptor Activation and Cell Proliferation

Wen Shi,^{*‡} Huizhou Fan,^{*‡} Lillian Shum,^{*‡} and Rik Derynck^{*‡}

^{*}Department of Growth and Development, and [‡]Department of Anatomy, Programs in Cell Biology and Developmental Biology, University of California at San Francisco, San Francisco, California 94143

Abstract. Transforming growth factor- α (TGF- α) is a member of the EGF growth factor family. Both transmembrane TGF- α and the proteolytically released soluble TGF- α can bind to the EGF/TGF- α tyrosine kinase receptor (EGFR) and activate the EGFR-induced signaling pathways. We now demonstrate that transmembrane TGF- α physically interacts with CD9, a protein with four membrane spanning domains that is frequently coexpressed with TGF- α in carcinomas. This interaction was mediated through the extracellular domain of transmembrane TGF- α . CD9 expression strongly decreased the growth factor- and PMA-induced proteolytic conversions of transmembrane to soluble TGF- α and strongly enhanced the TGF- α -induced EGFR activation, presumably in conjunc-

tion with increased expression of transmembrane TGF- α . In juxtacrine assays, the CD9-induced EGFR hyperactivation by transmembrane TGF- α resulted in increased proliferation. In contrast, CD9 coexpression with transmembrane TGF- α decreased the autocrine growth stimulatory effect of TGF- α in epithelial cells. This decrease was associated with increased expression of the cdk inhibitor, p21^{CIP1}. These data reveal that the association of CD9 with transmembrane TGF- α regulates ligand-induced activation of the EGFR, and results in altered cell proliferation.

Key words: transforming growth factor- α • CD9 • epidermal growth factor receptor • ectodomain shedding

Introduction

Transforming growth factor- α (TGF- α)¹ is a member of a family of structurally related growth factors that includes EGF, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (Bosenberg and Massagué, 1993). Similarly to these family members, TGF- α is synthesized as a transmembrane protein that can undergo a regulated cleavage of the ectodomain to release a soluble form of TGF- α (Bringman et al., 1987; Gentry et al., 1987). Both the transmembrane and the soluble forms are biologically active and able to activate the EGF receptor (EGFR), a transmembrane tyrosine kinase (Brachmann et al., 1989; Wong et al., 1989). The growth factor binds to the EGFR

through its 50-amino acid core sequence with a characteristically spaced six cysteine pattern that is found in all members of the TGF- α /EGF family (Derynck, 1992; Lee et al., 1995). After ligand binding, the receptor dimer undergoes autophosphorylation and, consequently, induces signaling cascades that lead to a mitogenic response and increased cell proliferation (Schlessinger and Ullrich, 1992). The best characterized signaling cascade, which is induced by the activated EGFR, goes through Ras and Raf and activates mitogen-activated protein (MAP) kinases, which induce transcription of various genes (Hill and Treisman, 1995; Treisman, 1996). In addition, EGFR activation also induces several other signaling cascades, such as the phosphatidylinositol-3 kinase pathway (Vanhaesebroeck et al., 1997) that activates small GTPases and cross-talks with the MAP kinase pathway (Vojtek and Cooper, 1995). Other members of the TGF- α /EGF family also activate the EGFR or structurally closely related tyrosine kinase receptors (Alroy and Yarden, 1997; Riese and Stern, 1998).

TGF- α is expressed by a variety of cell types from neuroectodermal origin, most commonly by epithelial cells in-

Address correspondence to Rik Derynck, Department of Growth and Development, University of California at San Francisco, San Francisco, CA 94143-0640. Tel.: (415) 476-7322. Fax: (415) 476-1499. E-mail: derynck@itsa.ucsf.edu

The present address for L. Shum is Craniofacial Development Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892-2745.

¹Abbreviations used in this paper: EGFR, EGF/TGF- α tyrosine kinase receptor; HB-EGF, heparin-binding EGF-like growth factor; MAP, mitogen-activated protein; TGF- α , transforming growth factor- α .

cluding keratinocytes. In these cells, the transmembrane TGF- α formed at the cell surface seems to be the predominant form of TGF- α . In vivo, transmembrane TGF- α is likely to interact in a juxtacrine or paracrine manner with the EGFR on neighboring cells, or in an autocrine manner on the same cells. TGF- α is also expressed in a large diversity of tumors, not only in tumors of neuroectodermal origin, but also in mesenchymally derived tumors (Derynck et al., 1987; Derynck, 1992; Lee et al., 1995). Carcinomas, which are derived from epithelial cells, consistently express relatively high levels of TGF- α . Although no controlled comparisons have been done, the expression of TGF- α is generally upregulated when an epithelial cell progresses into a carcinoma cell. In addition, the proteolytic cleavage of transmembrane TGF- α to release soluble TGF- α seems to be also induced or enhanced concomitantly with this transition. Since carcinomas also express EGFR, often at increased levels when compared with normal cells (Khazaie et al., 1993), TGF- α and EGFR activation are likely to regulate tumor cell proliferation and tumor development, a notion that is strongly supported by cell culture (Rosenthal et al., 1986; Di Marco et al., 1989) and animal experiments (Jhappan et al., 1990; Matsui et al., 1990; Sandgren et al., 1990).

In contrast to the well characterized ability of the activated EGFR to activate several signaling pathways, little is known about how the ligands are presented to the EGFR. Although transmembrane TGF- α potently activates the EGFR, its presentation at the cell surface and its interaction with the EGFR may be regulated by accessory proteins. Several putative transmembrane proteins have genetically been implicated in the activation of the EGFR by TGF- α -related proteins in *Drosophila* (Perrimon and Perkins, 1997), but little is known about their function at the cellular level and about proteins that regulate the presentation of TGF- α -related factors in vertebrates. Transmembrane TGF- α has been shown to interact with another transmembrane protein, p106, but its identity has not been reported (Shum et al., 1994). However, transmembrane HB-EGF has been shown to interact with a transmembrane protein, CD9 (Iwamoto et al., 1994), but no interaction with transmembrane TGF- α has been reported.

CD9 is a 24–27-kD cell surface protein with four predicted transmembrane domains that belong to the tetraspanin family. Originally identified as a surface antigen on lymphohematopoietic cells, CD9 is expressed by epidermal, basophil, pre-B cells, activated T cells, platelets, as well as neural cell lines (Maecker et al., 1997). CD9 is also expressed in most carcinomas, the tumor types that consistently express TGF- α and EGFR (Miyake et al., 1996; Cajot et al., 1997; Huang et al., 1998). However, little is known about the biological function of CD9 in tumor cells or any other cell type. CD9 has been shown to associate with several combinations of β 1 integrin complexes (Hemler, 1998) and increased CD9 expression can enhance the integrin-dependent cell motility of B cells (Shaw et al., 1995). In addition, CD9 has also been identified as DRAP27, a protein that enhances the affinity of the diphtheria toxin to its receptor, HB-EGF (Mitamura et al., 1992). The discovery that HB-EGF represents the diphtheria toxin receptor was in fact the basis for the observation that CD9 and HB-EGF

associate with each other (Iwamoto et al., 1994), and led to the observation that CD9 enhances the juxtacrine mitogenic activity of HB-EGF (S. Higashiyama et al., 1995). No observations have linked CD9 with the biology of TGF- α or a potential role for CD9 in carcinoma development. However, an inverse correlation has been found between CD9 expression in carcinomas and the prognosis for the behavior, invasiveness, and prognosis of the cancer (M. Higashiyama et al., 1995; Miyake et al., 1996; Cajot et al., 1997; Huang et al., 1998).

In this report, we demonstrate that transmembrane TGF- α and CD9 interact with each other, and that this interaction is mediated through the extracellular sequence of TGF- α . Increased CD9 expression decreases the growth factor-induced release of the TGF- α ectodomain and enhances the transmembrane TGF- α -induced EGFR stimulation. The increased EGFR activity in epithelial cells results in a high level of expression of the cdk inhibitor p21^{CIP1}, thus, decreasing the TGF- α -induced cell proliferation.

Materials and Methods

Plasmids

The mammalian expression plasmids pRK5 and pRK7 were previously described (Graycar et al., 1989). The expression plasmid pRK7-CD9-hygro was generated by inserting the full size CD9 coding sequence, subcloned as a HindIII-XbaI fragment from pRC/CMV/CD9 (Iwamoto et al., 1994), into pRK7-hygro. The XbaI-HindIII fragment was filled in with Klenow DNA polymerase I and subcloned into the filled-in HindIII site of pRK7-hygro. pRK7-hygro is a derivative of pRK7, in which the 1.7-kbp XhoI-XbaI fragment of pREP7 (Invitrogen Corp.) that encodes the hygromycin gene was inserted into the HpaI site of pRK7 by blunt end ligation.

The expression plasmids for full size TGF- α or the cytoplasmically truncated version (pRK7- $\alpha\Delta$ c) were previously described (Shum et al., 1994). Using PCR-based approaches, the full size TGF- α coding sequence was modified to encode the derivatives TGF- α -myc or TGF- $\alpha\Delta$ E. TGF- α -myc corresponds to the full size transmembrane TGF- α with the GGE-QKLISEEDLGG sequence, i.e., the myc epitope tag flanked by diglycines, inserted between amino acids 93 and 94. This location is immediately downstream the cleavage site for mature TGF- α and before the transmembrane segment. TGF- $\alpha\Delta$ E corresponds to the transmembrane TGF- α -myc from which the 50-amino acid mature TGF- α was removed. The TGF- α -myc and TGF- $\alpha\Delta$ E coding sequences were inserted into the EcoRI-HindIII sites of the pRK5 expression plasmid. pRK5-EGFR was generated by excising the XbaI-HindIII fragment from pXER (a gift from Dr. G. Gill, University of California, San Diego, CA) containing 3.5 kbp of EGFR cDNA, and inserting it into the corresponding sites of pRK5.

Cell Culture and Transfections

CHO cells were cultured in Ham's F12 medium supplemented with 10% FBS and 10 μ g/ml penicillin/streptomycin. 293 cells were cultured in DME medium, supplemented with 4.5 g glucose per liter, 10% FBS, and 10 μ g/ml penicillin/streptomycin. MDCK cells were grown in DME medium, supplemented with 4.5 g glucose per liter, 10% FBS, and 10 μ g/ml penicillin/streptomycin. WEHI-3B cells were cultured in RPMI 1640 medium supplemented with 15% FBS, 10 μ g penicillin/streptomycin. 32D cells and 32D-EGFR (EP170.7) cells (Pierce et al., 1988) were grown in suspension in RPMI 1640 medium with 10% FBS, 5% WEHI-3B conditioned medium. Stable transfected MDCK cell lines were maintained in the presence of 400 μ g/ml G418 (for neomycin resistance) or 200 μ g/ml hygromycin (for hygromycin resistance).

Stable transfected MDCK cells expressing TGF- α were obtained from Dr. R. Coffey (Vanderbilt University Medical School, Nashville, TN) (Dempsey and Coffey, 1994). These cells and the parental MDCK cells were transfected with pRK7-CD9-hygro using the calcium phosphate precipitation method (Ausubel et al., 1997). Transfected single colonies were

selected in the presence of 400 $\mu\text{g/ml}$ hygromycin, isolated 12 d after transfection, and screened for CD9 expression by immunofluorescence.

Metabolic Labeling, Coimmunoprecipitation, and Immunoblot Analysis

Metabolic labeling and coimmunoprecipitations were carried out essentially as described (Shum et al., 1994). In brief, the supernatants of the lysed 293, CHO, or MDCK cells were precleared with protein A-Sepharose (Pharmacia), and conjugated with rabbit anti-mouse IgG (Jackson ImmunoResearch Labs), for 1 h at 4°C. After centrifugation, the precleared supernatants were incubated at 4°C for 2 h with the anti-TGF- α mAb α 1 (5 $\mu\text{g/ml}$) (Bringman et al., 1987) or the anti-EGFR antibody Ab-5 (Calbiochem-Novabiochem) and protein A-Sepharose preloaded with rabbit anti-mouse IgG. The immunoprecipitated samples were washed three times with lysis buffer, incubated in SDS-Laemmli sample (Laemmli, 1970), and analyzed by SDS-PAGE. For cell surface immunoprecipitation of transmembrane TGF- α , cells were first incubated with the α 1 mAb (Bringman et al., 1987) for 2 h at 4°C, and then washed and lysed in Triton lysis buffer. The lysate was subjected to immunoprecipitation as described above.

For Western blotting, the immunoprecipitated protein membranes separated by SDS-PAGE, were electrotransferred onto nitrocellulose using a Mini Transblot Apparatus (Bio-Rad Laboratories). The immunoblots were pretreated with 5% BSA (for antiphosphotyrosine Western blots) or 5% dry milk (for the use of other antibodies) in TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween) for 1 h at room temperature. After three washes with TBST, antibody was added at a concentration of 1–10 $\mu\text{g/ml}$ in TBST, and the incubation proceeded overnight at 4°C. The blot was washed again three times and incubated with a 1:2,500-fold diluted HRP-conjugated goat anti-mouse IgG (Amersham Inc.) for 1 h at room temperature. The blots were washed again and developed using ECL substrates (Pierce Chemical Co.). Anti-TGF- α antibody was purchased from Calbiochem-Novabiochem, anti-CD9 and anti-p21 antibodies from PharMingen, and antiphosphotyrosine antibody from Transduction Laboratories.

Measurement of Transmembrane TGF- α Cleavage

The release of soluble TGF- α from transmembrane TGF- α was measured in transiently transfected CHO cells, as described previously (Fan and Derynck, 1999). In brief, CHO cells, grown in 6-well plates, were transfected using Lipofectamine (GIBCO BRL) with 0.2 μg pRK7-TGF- α with or without 1 μg pRK7-CD9-hygro or the control plasmid pRK7-hygro. Cells were switched into F12 medium, supplemented with 10% FBS, cultured for another 5 h, and then serum-starved overnight. To analyze the levels of cell-associated TGF- α , cells were pulse-labeled with [^{35}S]methionine/cysteine in F12 medium for 20 min, rinsed with medium, and incubated for 1 h at 37°C in F12/DME (1:1) medium, 0.1% BSA with or without 10% heat-inactivated FBS, or 10 nM PMA. Cells were again rinsed with cold medium and lysed in TBS containing 1% NP-40. Transmembrane TGF- α was immunoprecipitated from the cell extracts using the mAb α 1 (Bringman et al., 1987), the immunoprecipitates were analyzed by SDS-PAGE and autoradiography, and levels of transmembrane TGF- α were quantitated by ImageQuant software (Molecular Dynamics). To measure the levels of soluble TGF- α in the medium, [^{35}S]methionine/cysteine labeling was performed for 2 h. Soluble TGF- α , which was released into the medium, was analyzed by immunoprecipitation followed by gel analysis or liquid scintillation counting.

Immunofluorescence

Cells were grown in 4-well chamber slides (Falcon Plastics) and fixed with 3.7% formaldehyde in PBS for \sim 10 min. They were rinsed twice with PBS and permeabilized with 0.1% Triton-X 100, 1% BSA in PBS for 7 min, washed twice with PBS with a 5-min interval, and treated with 3% BSA in PBS for 30 min. After washing with PBS, the cells were incubated with the α 1 mAb (4 $\mu\text{g/ml}$) in 2% horse serum in PBS for 1 h at room temperature. The unbound antibody was removed by repeated washes in PBS. The cells were incubated with rhodamine (TRITC)-conjugated affinity-purified donkey anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, Inc.) (4 $\mu\text{g/ml}$) for 30–60 min. After washing the cells thoroughly with PBS, the slides were mounted with Gel-mount (Sigma Chemical Co.). All procedures were performed at room temperature. The fluorescence staining was examined with a Nikon fluorescence microscope.

Measurements of DNA Synthesis

Cells were plated at 2×10^4 cells/well in 12-well plates in normal culture medium and incubated overnight. 300 μl of serum-free medium containing 4 $\mu\text{Ci/ml}$ of [^3H]thymidine was added and incubation proceeded for 4 h at 37°C. After removal of the medium, the cells were washed twice with ice-cold 5% TCA and three times with ice-cold water. The cells were lysed with 250 μl 0.5 M NaOH, and the incorporated [^3H]thymidine in the cell lysates was determined using liquid scintillation counting.

Determination of Cell Numbers

MDCK cells were plated at 40% confluency in multiple 60-mm diam plates. At daily intervals after plating, one plate of cells was trypsinized and the cells were resuspended in 5 ml medium. The cells in a 10- μl sample were counted, and the total cell number was calculated as a function of time to determine the growth rate.

Measurement of Juxtacrine Growth Stimulation

CHO cells, which either express transmembrane TGF- α or not, were tested for their ability to stimulate proliferation of 32D-EGFR cells through cell-cell contact. The method was modified from S. Higashiyama et al. (1995). CHO cells were plated to 50% confluence in 12-well plates and incubated for 12 h at 37°C. The cells, either live or formalin-fixed, were washed with RPMI 1640 medium containing 10% FBS. 1×10^5 32D or 32D-EGFR cells were added in the same medium. After 24 h, [^3H]thymidine (1 $\mu\text{Ci/well}$ /500 μl) was added, and incubation proceeded for 4 h at 37°C. Subsequently, the 32D or 32D-EGFR cells were gently removed, collected on a nitrocellulose filter, and [^3H]thymidine incorporation was measured using liquid scintillation counting.

Results

Physical Interaction of CD9 with Transmembrane TGF- α

It has been reported that CD9 interacts with pro-HB-EGF, but not transmembrane TGF- α . Since transmembrane TGF- α and pro-HB-EGF both have a structurally related extracellular domain sequence with six conserved cysteines, we evaluated whether CD9 physically associates with transmembrane TGF- α . As shown in Fig. 1, A and B, CD9 coimmunoprecipitated with transmembrane TGF- α . No interaction was seen with another transmembrane protein, such as the type II TGF- β receptor (data not shown). In addition, CD9 did not interact with the EGFR, the receptor for TGF- α (Fig. 1 C).

To characterize the structural basis of this interaction, we evaluated the ability of CD9 to associate with several derivatives of transmembrane TGF- α . One of these, TGF- $\alpha\Delta\text{C}$, lacked the cytoplasmic domain but still associated with CD9, indicating that the cytoplasmic domain of transmembrane TGF- α is not required for this interaction (Fig. 2 A). To assess whether the extracellular domain of transmembrane TGF- α plays a role in the association with CD9, we also examined the interaction with a derivative of the transmembrane TGF- α , TGF- $\alpha\Delta\text{E}$. In TGF- $\alpha\Delta\text{E}$, the 50-amino acid core sequence of fully processed TGF- α was replaced with a Myc epitope tag. CD9 was unable to associate with this derivative of transmembrane TGF- α (Fig. 2 A), suggesting that the 50-amino acid core TGF- α sequence is required for this interaction. In control experiments, CD9 still coprecipitated with a derivative of transmembrane TGF- α in which the 50-amino acid TGF- α core was followed by the Myc epitope sequence (data not shown), strongly suggesting that the Myc antibody does

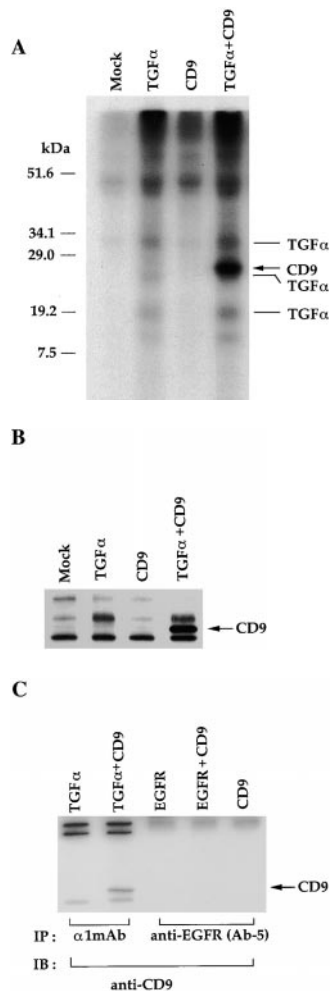


Figure 1. Association of CD9 with transmembrane TGF- α . (A) Anti-TGF- α immunoprecipitates of ^{35}S -labeled, transfected 293 cell lysates were analyzed using SDS-PAGE. Cells were transfected with control pRK5 plasmid (lane 1), pRK7-TGF- α (lane 2), pRK7-CD9 (lane 3), and pRK7-TGF- α and pRK7-CD9 (lane 4). The previously characterized three transmembrane forms of TGF- α (Bringman et al., 1987) are marked. CD9 is marked with an arrow. As shown, CD9 coprecipitates with transmembrane TGF- α . (B) Anti-TGF- α immunoprecipitates of unlabeled, transfected 293 cells were analyzed by Western blotting using an anti-CD9 antibody. Cells were transfected with control pRK5 plasmid (lane 1) or with expression plasmids for TGF- α and/or CD9, as marked (lanes 2–4). CD9 is marked with an arrow, whereas nonspecific bands are not marked. (C) CD9 does not associate with the EGFR. Anti-TGF- α or anti-EGFR immunoprecipitates of unlabeled, transfected CHO cells were analyzed by Western blotting using an

anti-CD9 antibody. Cells were transfected with expression plasmids for TGF- α and/or CD9, as marked. CD9 is marked with an arrow, whereas nonspecific bands are not marked.

not interfere with the association with CD9. Finally, expression of HB-EGF decreased the level of CD9 that associated with transmembrane TGF- α (Fig. 2 B), strongly suggesting that transmembrane HB-EGF and TGF- α compete with each other for interaction with CD9. This observation further supports the notion that the 50-amino acid core TGF- α sequence is essential for association with CD9, since the sequence similarity of transmembrane HB-EGF and TGF- α is only apparent in this core segment.

CD9 Coexpression Inhibits Growth Factor- and PMA-induced Transmembrane TGF- α Cleavage

The interaction of CD9 with the extracellular domain of transmembrane TGF- α raises the possibility that CD9 association regulates the cleavage of the ectodomain of transmembrane TGF- α . This cleavage occurs immediately downstream from the 50-amino acid core sequence through the action of a cell surface-associated metalloprotease (Arribas et al., 1996), and results in the release of the soluble growth factor. In cell culture, TGF- α -expressing cells undergo a basal level of soluble TGF- α release, which

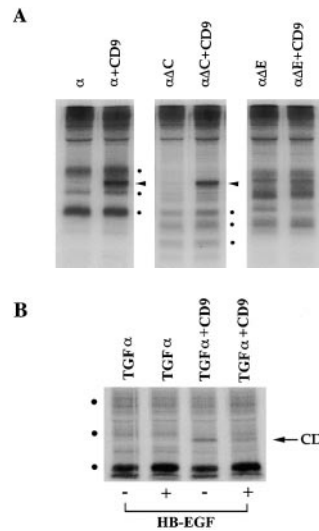


Figure 2. CD9 interacts with the extracellular domain of transmembrane TGF- α . (A) Interaction of CD9 with deletion mutants of transmembrane TGF- α . Anti-TGF- α immunoprecipitates of ^{35}S -labeled, transfected 293 cell lysates were analyzed using SDS-PAGE. Cells were transfected with expression plasmids for transmembrane TGF- α (α), a cytoplasmically truncated transmembrane TGF- α ($\alpha\Delta\text{C}$) or a transmembrane TGF- α mutant lacking the extracellular TGF- α core sequence ($\alpha\Delta\text{E}$), with or without an expression plasmid for CD9. The arrowheads show CD9, which co-

precipitates with transmembrane forms of wild-type TGF- α or TGF- $\alpha\Delta\text{C}$ but not with TGF- $\alpha\Delta\text{E}$. The bullets show the three transmembrane TGF- α forms, which in the case of $\alpha\Delta\text{C}$ and $\alpha\Delta\text{E}$ are predictably smaller in size than in wild-type TGF- α . (B) Coexpression of HB-EGF competes with binding of transmembrane TGF- α to CD9. CHO cells were transfected with expression plasmids for TGF- α with or without CD9, in the presence or absence of transmembrane HB-EGF coexpression. Immunoprecipitation of transmembrane TGF- α resulted in coprecipitation of CD9, as shown in lane 3, and the level of coprecipitated CD9 was considerably less, when HB-EGF was coexpressed (lane 4). In this experiment, the expression of the three transmembrane TGF- α forms (marked with bullets) is considerably less than in Fig. 1 A.

is controlled by the p38 MAP kinase signaling cascade, whereas growth factor receptor-stimulated TGF- α ectodomain release (e.g., in response to EGFR activation) is mediated through the Erk/MAP kinase pathway (Fan and Derynck, 1999). Therefore, we evaluated the ability of serum, a source of growth factors such as PDGF, to induce ectodomain cleavage and consequent release of soluble TGF- α . As shown in Fig. 3, the serum- and PMA-induced release of soluble TGF- α , as assessed by the levels of both the soluble TGF- α in the medium and the cell surface-associated transmembrane TGF- α , were strongly inhibited by CD9. These results suggest that the interaction of CD9 with transmembrane TGF- α strongly decreases its susceptibility to ectodomain cleavage.

Increased EGFR Activation in Cells Coexpressing Transmembrane TGF- α and CD9

To assess the physiological effects of CD9 on the activity of transmembrane TGF- α , we expressed CD9, TGF- α , or both in CHO cells, which lack endogenous EGFR, and in MDCK cells, which express EGFR endogenously. Stably transfected CHO cells were tested for their ability to stimulate EGF receptors on transfected 32D cells, a hematopoietic cell line that lacks endogenous EGFR and the related HER-2, -3, and -4 receptors, but was transfected to express EGFR (Pierce et al., 1988). Direct cell contact between CHO cells expressing transmembrane TGF- α and

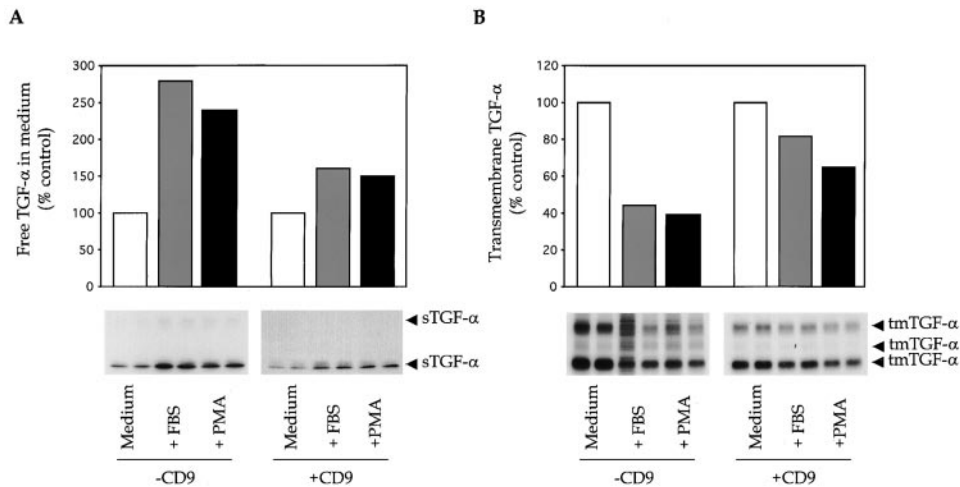


Figure 3. CD9 expression inhibits the serum- or PMA-induced proteolytic release of soluble TGF- α . (A) CD9 expression decreases the serum- or PMA-induced release of soluble TGF- α into the medium. The bottom panel shows the soluble TGF- α in the medium; the smaller form is the 50-amino acid form, whereas the larger one, which is much lighter in intensity, is the glycosylated form. The top panel shows the average values of duplicate samples, based on liquid scintillation counting of the immunoprecipitated TGF- α in the medium. The amounts of free TGF- α released

into the medium were normalized to the 100% value for the unstimulated control samples (B) The serum- or PMA-induced processing of transmembrane TGF- α is decreased in the presence of CD9. Transmembrane TGF- α was immunoprecipitated from the lysates and visualized by SDS-PAGE and autoradiography (bottom). As shown, serum or PMA induced ectodomain cleavage and a resulting decrease of transmembrane TGF- α . CD9 inhibited this decrease in cell-associated TGF- α . The top panel shows a quantitation of the cell-associated TGF- α by phosphorimage analysis of the immunoprecipitated cell-associated TGF- α . All experiments were carried out in duplicate, and the values were averaged and normalized to the 100% value for the unstimulated control samples.

32D-EGFR cells allowed transmembrane TGF- α to stimulate the tyrosine autophosphorylation of the EGFR, a measure of juxtacrine receptor activation induced by TGF- α . In contrast, cells expressing only CD9 did not stimulate EGFR autophosphorylation. Coexpression of CD9 with transmembrane TGF- α , however, resulted in a higher level of EGFR receptor activation than TGF- α alone (Fig. 4 A). This was observed using both live CHO

cells (Fig. 4 A) or fixed CHO cells, which do not have the ability to release soluble TGF- α (data not shown), as donor cells.

We also generated stably transfected MDCK cells, which express TGF- α , CD9, or both. Like most epithelial cells, MDCK cells make low levels of endogenous TGF- α and EGFR, and the autocrine stimulation of the EGFR by TGF- α is thought to be important for epithelial cell proliferation (Tsao et al., 1996). The expression levels of TGF- α and CD9 were considerably lower than in the transfected CHO cells (data not shown). Using these stable cell lines, we evaluated the level of EGFR tyrosine phosphorylation as a measure of autocrine, TGF- α -induced receptor activation. The MDCK cells that coexpressed TGF- α and CD9 showed a higher level of EGFR activation than the cells expressing only TGF- α (Fig. 4 B). This result is consistent with the higher level of juxtacrine EGFR stimulation of 32D cells by TGF- α /CD9 coexpressing CHO cells, when compared with TGF- α -expressing cells (Fig. 4 A). The low level of EGFR phosphorylation in the control-transfected MDCK cells or the MDCK cells expressing CD9 alone is most likely due to stimulation by endogenous TGF- α .

We next evaluated whether the increased EGFR stimulation was due to an increased ability of transmembrane TGF- α to activate the receptor and/or an increased number of transmembrane TGF- α proteins at the cell surface. As shown in Fig. 5 A, coexpression of CD9 resulted in increased expression of transmembrane TGF- α both at the cell surface of stably transfected CHO cells (Fig. 5 A) and in total cell lysates of the MDCK cells (data not shown). This increased expression of transmembrane TGF- α , when CD9 was coexpressed, was consistently seen in multiple stably transfected CHO (data not shown) and MDCK cell clones (Fig. 5 B). Immunofluorescence staining of permeabilized MDCK cells also demonstrated increased trans-

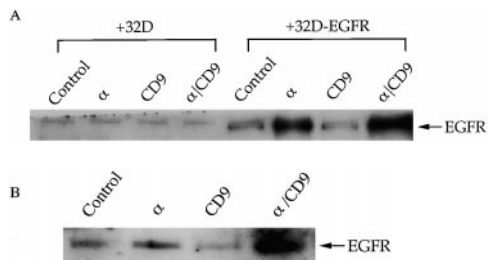


Figure 4. Increased EGFR tyrosine phosphorylation in response to transmembrane TGF- α /CD9 costimulation. (A) 32D cells or EGFR expressing 32D cells (32D-EGFR) were brought in contact with CHO cells expressing transmembrane TGF- α , CD9, or both, as shown. EGFR tyrosine phosphorylation in the 32D-EGFR cells was assessed by antiphosphotyrosine Western blotting reactivity of the 170-kD EGFR band using equal amounts of cell lysate protein. The autoradiographic intensity of the phosphorylated EGFR, induced by TGF- α /CD9 coexpressing cells (α /CD9), was 1.33-fold higher than the EGFR phosphorylation, induced by TGF- α -expressing cells (α), as assessed by scanning of the autoradiogram. (B) Tyrosine phosphorylation of endogenous EGFR in MDCK cells, stably transfected with expression plasmids for transmembrane TGF- α , CD9, or both. EGFR tyrosine phosphorylation was assessed as in A. Scanning revealed a 1.42-fold higher intensity of the phosphorylated EGFR band in TGF- α /CD9 coexpressing cells (α /CD9), when compared with the cells that only overexpress TGF- α (α).

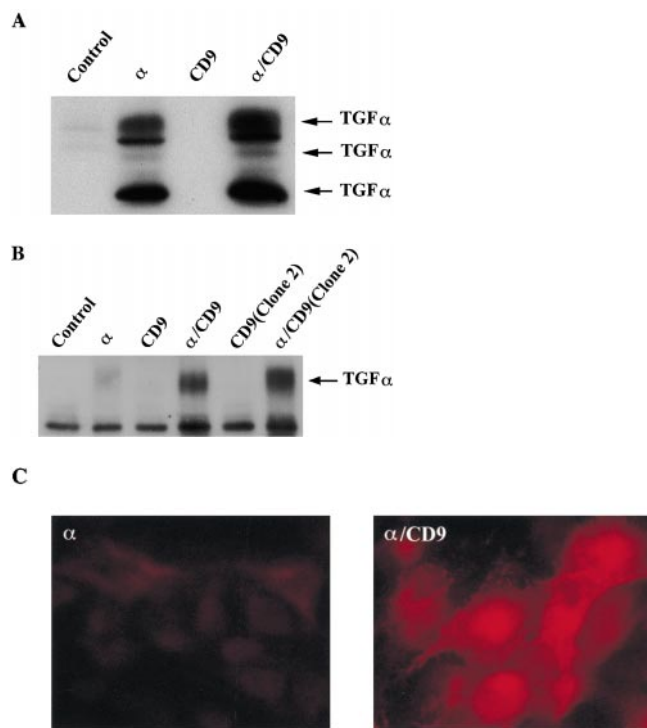


Figure 5. CD9 coexpression enhances the cell surface presentation of transmembrane TGF- α . (A) Cell surface expression of transmembrane TGF- α in CHO cells, stably transfected with expression plasmids for TGF- α , CD9, or both. Intact cells were incubated with the anti-TGF- α mAb α 1, before lysis and anti-TGF- α immunoprecipitation, followed by anti-TGF- α Western blotting using Ab-1 antibody (Calbiochem-Novabiochem). The three forms of transmembrane TGF- α are marked. The nature of the sharp, unmarked band is not known, but may be due to an artifact in gel migration. Scanning of the upper and lower TGF- α band on the autoradiogram revealed a 1.68- and 1.24-fold higher intensity, respectively, in the TGF- α /CD9 coexpressing cells (α /CD9) when compared with the cells that only overexpress TGF- α (α). (B) CD9 coexpression results in enhanced levels of transmembrane TGF- α in MDCK clones. Besides the clone, marked α /CD9, which was used in further experiments, another clone (clone 2) also showed enhanced TGF- α levels when compared with the TGF- α overexpressing MDCK cells (marked α). Control marks the parental MDCK cells, which did not overexpress TGF- α or CD9, whereas CD9 designates MDCK cells that overexpress CD9 but not TGF- α . Transmembrane TGF- α was assessed by immunoprecipitation with anti-TGF- α antibody, followed by Western blot using anti-TGF- α antibody. The TGF- α form shown corresponds to the large glycosylated form. The non-specific band was detectable in all lanes. (C) Immunostaining of transmembrane TGF- α in permeabilized MDCK cells, stably transfected with an expression plasmid for TGF- α in the absence (left) or presence (right) of overexpressed CD9. Immunoprecipitation of ^{35}S -labeled cell lysates confirmed the higher expression of transmembrane TGF- α in TGF- α /CD9 coexpressing cells, which, based on scanning of the autoradiogram, was 4.4-fold higher than in the cells that only overexpress TGF- α (data not shown).

membrane TGF- α not only at the cell surface, but also in the perinuclear region (Fig. 5 C). The increase in juxtacrine and autocrine EGFR activation in 32D and MDCK cells, respectively, is therefore not necessarily a direct con-

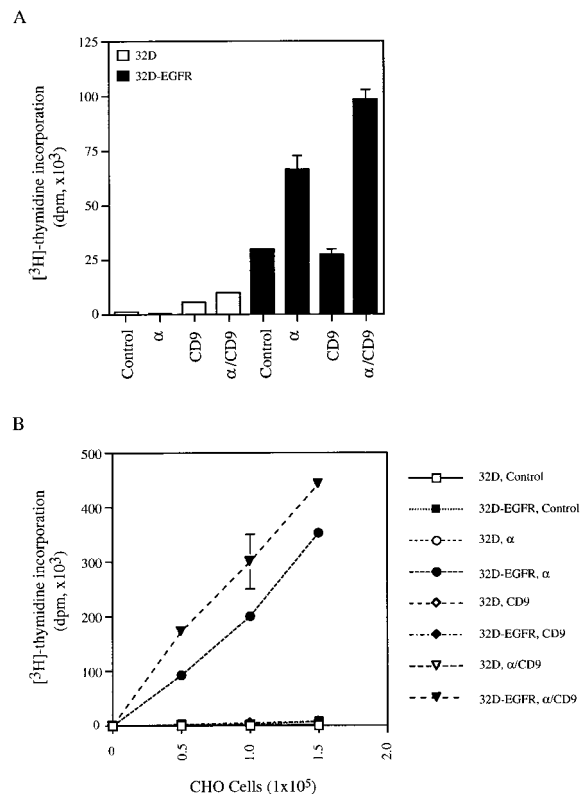


Figure 6. CD9 increases the juxtacrine transmembrane TGF- α -induced DNA synthesis. (A) 32D cells, either parental or EGFR-expressing cells, were laid on top of a confluent monolayer of live CHO cells expressing TGF- α , CD9, or both (as marked), and [^3H]dT incorporation in the 32D cells was measured. The values, shown as bar graphs, represent the averages of triplicates in two independent experiments with their SEM. (B) Cell number-dependent, juxtacrine stimulation of DNA synthesis. The experiments were done as in A, except that the 32D cells were incubated with different numbers of fixed CHO cells, as shown in the abscissa. Only the EGFR expressing 32D cells were stimulated by TGF- α -expressing CHO cells, and this stimulation was higher when TGF- α was coexpressed with CD9. None of the other cell combinations showed stimulation. In several cases, the SEM values are too low to be visualized in the diagram.

sequence of the interaction of CD9 with transmembrane TGF- α per se, but may result from the increased transmembrane TGF- α levels at the surface of cells that coexpress CD9.

Coexpression of CD9 Enhances the Juxtacrine, but Decreases the Autocrine Mitogenic Activity of Transmembrane TGF- α

We next assessed the effect of CD9 expression on the ability of transmembrane TGF- α to stimulate the EGFR-induced DNA synthesis and cell proliferation. Coculture of cells expressing transmembrane TGF- α with EGFR-expressing 32D cells allowed us to measure the juxtacrine activity of transmembrane TGF- α . CHO cells, which lack endogenous TGF- α and EGFR did not induce [^3H]dT incorporation in the 32D-EGFR cells, nor did TGF- α -expressing CHO cells stimulate the DNA synthesis in

parental 32D cells, which lack EGFR expression. In addition, expression of CD9 alone did not affect the DNA synthesis in EGFR-expressing 32D cells either. In contrast, TGF- α -expressing CHO cells stimulated DNA synthesis in EGFR-expressing 32D cells, and this stimulation was substantially increased when CD9 was coexpressed with transmembrane TGF- α . This was demonstrated using both live (Fig. 6 A) or fixed donor CHO cells (Fig. 6 B). The basis for the higher level of DNA synthesis in control- or CD9-stimulated 32D-EGFR cells, when compared with the parental 32D cells (Fig. 6 A), is unclear but may be related to a background activity of the EGFR in the absence of exogenous stimulation.

Consistent with the ability of transmembrane TGF- α to activate the EGFR in 32D cells, the TGF- α -expressing MDCK cells had a higher rate of DNA synthesis and cell proliferation than control-transfected MDCK cells (Fig. 7, A and B). This observation is also consistent with the growth stimulatory activity of soluble TGF- α on these cells (data not shown) and many other cell lines. The rate of DNA synthesis and cell proliferation of CD9-expressing cells was comparable to the control cells. In contrast to the TGF- α -expressing MDCK cells, coexpression of CD9 and transmembrane TGF- α resulted in a lower rate of DNA synthesis (Fig. 7 A) and cell proliferation (Fig. 7 B). This effect of CD9 was remarkable since the expression level of transmembrane TGF- α and the level of EGFR activation were considerably higher in these cells than in the TGF- α -expressing MDCK cells. Such a growth inhibitory activity could not be mimicked by addition of high levels of soluble TGF- α to these cells (data not shown). This result is also in contrast with the ability of coexpressed CD9 to enhance the juxtacrine, TGF- α -induced stimulation of DNA synthesis in 32D cells (Fig. 6 A). Furthermore, the expression level of transmembrane TGF- α in the CHO cells that were used in the juxtacrine stimulation assays, was considerably higher than in MDCK cells used in the autocrine stimulation (data not shown).

These results indicate that CD9 coexpression with transmembrane TGF- α results in increased transmembrane TGF- α levels at the cell surface and increased activation of the EGFR both in a juxtacrine (32D cells) and autocrine (MDCK cells) manner. Cells expressing both CD9 and TGF- α induced an enhanced juxtacrine stimulation of DNA synthesis and proliferation of 32D-EGFR cells. However, the strong increase in EGFR activation in MDCK cells, expressing both CD9 and TGF- α , resulted in growth inhibition, when compared with MDCK cells expressing only transmembrane TGF- α .

The CD9-induced Growth Inhibition in Transmembrane TGF- α -Expressing Cells Correlates with Increased p21^{CIP1} Expression

Although moderate activation of Raf, an effector of Ras signaling and stimulator of MAP kinase activation, results in stimulation of cell proliferation, a high level activation of Raf (Sewing et al., 1997; Woods et al., 1997) or Ras (Pumiglia and Decker, 1997; Olson et al., 1998) has been shown to stimulate the expression of the cdk inhibitor, p21^{CIP1}. When stimulated beyond a certain threshold level, p21^{CIP1} is able to induce growth arrest. Since Ras, Raf and

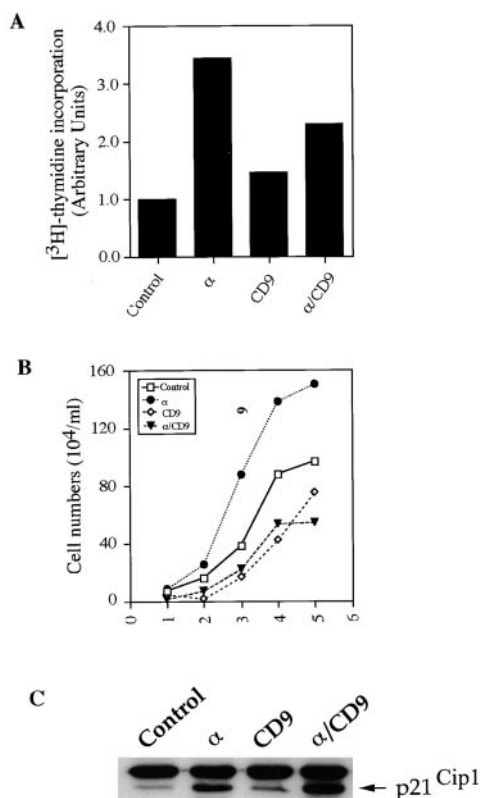


Figure 7. CD9 decreases the autocrine transmembrane TGF- α -induced DNA synthesis. (A) DNA synthesis rates in stably transfected MDCK cells. [3H]dT incorporation in transfected MDCK cells expressing TGF- α , CD9, or both, was measured as in Fig. 6. The values, shown as bar graphs, represent the averages of triplicates in two independent experiments with their SEM, which are too small to be seen in this experiment. (B) Cell proliferation, as measured by cell numbers, in stably transfected MDCK cells, overexpressing TGF- α , CD9, or both. The SEM values are too low to be visualized in the diagram. (C) Endogenous expression of p21^{CIP1} in transfected MDCK cells, expressing TGF- α , CD9, or both, as assessed by immunoprecipitation followed by Western blot analysis.

MAP kinase signaling are activated in response to EGF receptor activation (Schlessinger and Ullrich, 1992), we examined whether an increase in p21^{CIP1} expression could be the basis of the growth inhibition of the MDCK cells, which coexpress CD9 and transmembrane TGF- α .

As shown in Fig. 7 C, the level of p21^{CIP1} expression was considerably higher in MDCK cells, which coexpress transmembrane TGF- α and CD9, when compared with parental MDCK cells or the TGF- α -expressing MDCK cells. Cells expressing TGF- α alone also had enhanced p21^{CIP1} expression, albeit to a considerably lower level than in cells coexpressing CD9 and TGF- α . Thus, the level of p21^{CIP1} expression correlated with the level of EGFR activation (Fig. 4 B). Furthermore, the high level of p21^{CIP1} and its role as an inducer of growth arrest also correlated with the lower level of proliferation of the TGF- α /CD9 coexpressing cells, when compared with cells expressing TGF- α alone. The levels of p21^{CIP1} in these MDCK cell lines correlated with the transcription levels

from the p21^{CIP1} promoter, as assessed using a p21^{CIP1} promoter-luciferase reporter, transfected into the MDCK stable cells (data not shown). These results show that the high level of EGFR activation, induced by coexpression of transmembrane TGF- α and CD9, results in a high level of p21^{CIP1} expression in MDCK cells. This increase in p21^{CIP1} expression, a CDK inhibitor that can induce growth arrest when expressed beyond a certain threshold, may explain why cells that coexpress TGF- α and CD9 proliferate at a lower level than cells expressing TGF- α alone, even though they have a higher level of EGFR activation.

Discussion

TGF- α and related growth factors are expressed as transmembrane proteins and bind to and activate the EGFR, both as transmembrane proteins and as proteolytically released, soluble ligands (Derynck, 1992; Lee et al., 1995). EGFR-induced signaling leads to the expression of various genes and a proliferative response. The mechanisms of signal propagation through these and other EGFR-activated pathways are well characterized (Schlessinger and Ullrich, 1992; Moghal and Sternberg, 1999). In contrast, little is known about how the ligands are presented to the EGFR. Although transmembrane TGF- α activates the EGFR (Brachmann et al., 1989; Wong et al., 1989), its presentation at the cell surface and its interaction with the EGFR may be regulated by accessory proteins. We have evaluated the ability of CD9, a membrane protein with four predicted transmembrane segments, to associate with transmembrane TGF- α .

CD9 can associate with the transmembrane forms of HB-EGF and amphiregulin, and enhances their juxtacrine proliferative effect (S. Higashiyama et al., 1995; Inui et al., 1997). However, no interaction was observed between CD9 and TGF- α , and no effect of CD9 on TGF- α -stimulated DNA synthesis was seen in EGFR-expressing cells (S. Higashiyama et al., 1995; Inui et al., 1997). These data, together with peptide competition experiments, led to the conclusion that CD9 interacts with the heparin-binding domain of HB-EGF or amphiregulin, and not with transmembrane TGF- α , which lacks such a domain (Sakuma et al., 1997). We now show that CD9 does associate with transmembrane TGF- α , and that this interaction requires the extracellular 50-amino acid core sequence of TGF- α . Accordingly, CD9 does not interact with the $\alpha\Delta E$ mutant that lacks the TGF- α core sequence. In addition, transmembrane HB-EGF and TGF- α , which have sequence similarity only in their EGF/TGF- α -like core sequence, compete with each other for association with CD9. The interaction of CD9 with TGF- α is most likely mediated through the second and largest extracellular loop of CD9, which has been implicated in the association with HB-EGF.

CD9 and several other tetraspanins can associate with several types of integrins (Berdichevski et al., 1996; Hemler, 1998), and complexes of HB-EGF with CD9 and the $\alpha 3\beta 1$ integrin dimer have been observed (Nakamura et al., 1995). Therefore, CD9 may play a role in cell surface presentation of transmembrane TGF- α and, through the formation of oligomeric complexes with integrins, direct its localization to sites of cell contact or cell adhesion. In this

way, CD9 could facilitate and increase the efficiency of the TGF- α -induced stimulation of the EGFR, which has also been localized at these sites. Tetraspanins, such as the CD9-related CD151, CD63, and CD81, can also regulate other types of intracellular signaling. CD63, CD81, and CD151 recruit phosphatidylinositol 4-kinase toward the integrin-tetraspanin complex (Berdichevski et al., 1997; Yauch et al., 1998), and some protein kinase C isoforms can be recruited into complexes of CD81 and CD151 with integrins (Hemler, 1998). These observations raise the possibility that the interaction of transmembrane TGF- α with CD9 may mediate other signaling events, which await further characterization.

The interaction of CD9 with transmembrane TGF- α strongly decreased the serum- or PMA-induced ectodomain cleavage of TGF- α and consequent release of soluble TGF- α . This observation may have considerable significance. EGFR stimulation by e.g., TGF- α itself normally induces the release of soluble TGF- α , thus, decreasing the level of transmembrane TGF- α (Baselga et al., 1996; Fan and Derynck, 1999). Whereas soluble EGF or TGF- α induces internalization of the ligand-receptor complex and consequent attenuation of the response, binding of transmembrane TGF- α to EGFR is unlikely to do so, and is expected to result in a high level, sustained activation of EGFR. This would result in a much stronger EGFR response, as in internalization-defective EGFR mutants (Chen et al., 1989). Thus, expression of CD9 may inhibit the autocrine feedback mechanism, which normally results in TGF- α ectodomain cleavage and consequent attenuation of EGFR signaling, and allow transmembrane TGF- α to sustain signaling through the EGFR.

The much stronger stimulation of the EGFR induced by coexpressing CD9 and TGF- α , when compared with TGF- α expression alone, was evident from its increased tyrosine phosphorylation. This was observed both in an autocrine system, whereby TGF- α and CD9 were coexpressed in the same MDCK cells as the EGFR, and in juxtacrine assays, whereby the CD9/TGF- α -expressing cells were in contact with the EGFR on adjacent cells. The increased EGFR activation could be explained by inhibition of the TGF- α ectodomain cleavage and consequent sustained signaling by transmembrane TGF- α , combined with the higher levels of uncleaved TGF- α at the cell surface. In addition, CD9 coexpression may also increase the stability and half-life of transmembrane TGF- α , and increased EGFR stimulation may enhance the expression of transmembrane TGF- α , as described previously (Coffey et al., 1987; Tang et al., 1997). Whether the increase in transmembrane TGF- α levels in the presence of CD9 fully accounts for the increased EGFR activation is unclear, and additional effects of CD9 on the presentation of transmembrane TGF- α to the EGFR (e.g., by increasing its affinity or concentrating it at the cell surface in clusters) may be envisioned.

The effect of CD9 on the cell surface levels of the transmembrane growth factor may also explain why CD9 expression enhanced the juxtacrine mitogenic stimulation of transmembrane HB-EGF (S. Higashiyama et al., 1995). This would be consistent with the observed increase in diphtheria toxin binding to cells that coexpress CD9 with HB-EGF, the diphtheria toxin receptor, and the increase

in diphtheria toxin receptor binding sites on these cells (Mitamura et al., 1992; Iwamoto et al., 1994). In contrast, no major effect of CD9 on the cell surface levels of HB-EGF, as assessed using an anti-HB-EGF antibody, was detected (Iwamoto et al., 1994).

Consistent with the increased EGFR stimulation, CD9 enhanced the DNA synthesis in 32D cells, when compared with the effect of transmembrane TGF- α alone. This higher juxtacrine mitogenic response resembled the effect of CD9 on the activity of HB-EGF in a similar assay (Higashiyama et al., 1995). In contrast, the autocrine mitogenic activity of transmembrane TGF- α in MDCK cells was decreased in the presence of CD9, even though the level of EGFR activation (and TGF- α expression) was much higher than in cells expressing TGF- α alone. EGFR-induced growth inhibition has previously only been shown in squamous carcinoma cells, such as A431 cells, with a strongly amplified level of EGFR expression. In A431 cells, very low EGF levels induce a moderate proliferative response, and the more commonly used EGF concentrations induce growth inhibition (Kawamoto et al., 1984). Thus, hyperstimulation of EGFR signaling because of extremely high EGFR levels can induce growth inhibition (Kawamoto et al., 1984; MacLeod et al., 1986), presumably from hyperstimulation of the Raf/MAP kinase signaling cascade (Sewing et al., 1997; Woods et al., 1997) and Stat signaling (Bromberg et al., 1998). Our data now show that, in contrast to A431 cells, EGFR hyperstimulation can lead to growth inhibition at the normal physiological EGFR levels in epithelial cells, and that this can be achieved by coexpressing CD9 and transmembrane TGF- α .

The autocrine growth inhibitory effect of CD9 in TGF- α -expressing MDCK cells is in contrast with its stimulatory effect in juxtacrine assays using 32D cells. This difference in response does not correlate with higher EGFR levels in MDCK versus 32D cells, as could have been invoked based on the findings in A431 cells. Instead, the endogenous EGFR levels in MDCK cells are physiological and lower than in EGFR-transfected 32D cells, and the TGF- α levels in the transfected MDCK cells are lower than in the transfected CHO cells, which were used in the juxtacrine assays (data not shown). The growth inhibitory effect of TGF- α /CD9 in an autocrine context versus the stimulatory effect in juxtacrine assays may reflect a qualitative difference between these two modes of presentation of TGF- α /CD9 to the EGFR. Alternatively or in addition, this difference may also be due to cell type differences between the 32D cells of hematopoietic origin and the epithelial MDCK cells.

The autocrine growth inhibitory effect of CD9 in TGF- α -expressing MDCK cells was associated with a high expression level of p21^{CIP1}. p21^{CIP1} binds to cdk4 and cdk2 complexes, and increased p21^{CIP1} expression inhibits cyclin D- and cyclin E-dependent kinase activities and induces growth inhibition (Morgan, 1995; el-Deiry, 1998). The growth inhibitory effect of CD9 in TGF- α -expressing MDCK cells is, therefore, likely to be a direct consequence of the induction of p21^{CIP1}. Accordingly, the growth inhibitory effect of EGF on A431 cells with their extremely high EGFR levels is also paralleled by an induction of p21^{CIP1} (Fan et al., 1995). Thus, p21^{CIP1} expression and growth inhibition can be induced by EGFR hyper-

stimulation, either at highly amplified EGFR levels, such as in A431 cells, or at normal physiological EGFR numbers, when CD9 is coexpressed with transmembrane TGF- α . Induction of p21^{CIP1} as a result of EGFR stimulation may also explain the decreased proliferation of cells expressing transmembrane HB-EGF, when compared with cells expressing soluble HB-EGF (Miyoshi et al., 1997).

Finally, the association of CD9 with transmembrane TGF- α and its effect on EGFR signaling may play a role in the development of carcinoma tumors. When compared with normal epithelial cells, carcinomas, especially squamous carcinomas, frequently show increased TGF- α (Derynck et al., 1987; Derynck, 1992; Lee et al., 1995) and EGFR expression, and consequently high levels of EGFR activation (Khazaie et al., 1993). Increased TGF- α levels and autocrine EGFR activation are known to contribute to carcinoma formation and proliferation in mice (Jhappan et al., 1990; Matsui et al., 1990; Sandgren et al., 1990), thus, suggesting that increased TGF- α and EGFR numbers may contribute to carcinoma development in humans. The frequent expression of CD9 in carcinomas (Higashiyama et al., 1997; Adachi et al., 1998), combined with our current findings, now raises the possibility that CD9 could enhance autocrine, TGF- α signaling at normal EGFR levels (i.e., without the need for increased EGFR levels) and, in this way, regulate the behavior of carcinomas in vivo. A few studies have correlated the expression of CD9 with the clinical prognosis of carcinomas (Miyake et al., 1996; Cajot et al., 1997; Huang et al., 1998). Although these correlations have not been unambiguous, increased CD9 expression may correlate with a better prognosis of several carcinoma types in humans. This could be due in part to the decreased motility of cells with higher CD9 levels (Higashiyama et al., 1995; Miyake et al., 1996), and to physiological consequences of the interaction of CD9 with integrins. However, our results suggest that CD9 expression may also affect tumor development through its ability to regulate the TGF- α -induced activation of EGFR signaling and, consequently, cell proliferation.

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